In vitro Shoot Regeneration Improvement with Thidiazuron and Benzyladenine in Rapeseed (*Brassica napus* L.)

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Abstract
An *in vitro* method was developed for high frequency shoot regeneration and continuous production of *Brassica napus* L. in large number. The different concentrations of benzyladenine (1.5, 3.0 and 4.5 $mgl^{-1)}$ and thidiazuron (0, 0.15 and 0.30 mgl^{-1}) were evaluated for shoot and root regeneration using hypocotyl explants with 7, 14 and 21 days old. Shoot regeneration (SR) was affected by both benzyladenine (BA) and thidiazuron (TDZ). Maximum shoots were regenerated with 4.5 mgl^{-1} BA and 0.30 mgl^{-1} TDZ. Shoot regeneration was highly affected by age of explants, however, 14-day-old explants were more profitable for shoot development than others. In BA concentrations, the shoot regeneration declined with the increase of age of explants. Significant differences were found in interactions of BA and TDZ concentrations and ages of explants. The maximum root regenerating plants were obtained from using of 3 mgl^{-1} BA, 0.3 mgl^{-1} TDZ and 21 days old explants. Rooting of regenerating shoot occurred readily on indolebutyric acid supplemented media. Rooted plantlets were successfully established in soil and developed normal fertile flowers and viable seeds.

Keywords: tissue culture-cytokinin- hypocotyl- age of explant

Introduction

Brassica napus L. is an important oil seed crop worldwide. For improvement of yield and quality of different Brassica species, the classical methods of plant breeding are being supplemented with *in vitro* techniques and genetic manipulation. Thus, *in vitro* regeneration response in terms of number of shoot produced is an important factor determining the success of transformation. The different plant growth regulator such as benzyladenine (BA), 2,4-D, thidiazuron (TDZ) and indolebutyric acid (IBA) are used for plant regeneration *in vitro*.

TDZ is commonly used as a cotton defoliant agent. It aids in rapid regeneration of numbers of plant species (Eapen et al. 1998). In 1982 Mok et al. discovered the cytokinin - like activity of TDZ.The first reports described callus – promoting activity of TDZ in *Phaseolus lunatus* L. and *Glycine max L.*, and induction of plant regeneration in *Nicotiana tubacum*.

Thidiazuron is a substitute phenylurea. Although the biochemical action of the phenylurea is not completely understood, It's believed that it function to regulate metabolism of purine cytokinin or act directly as cytokinin or in concern with cytokinins (Mok et al., 1982). The biological activity of TDZ is higher than comparable to that of the most active adenin-type cytokinins (Mok et al.,1987; Bhagwat et al., 1996).

In order to increase the yield of shoot regeneration, we explored the organogenetic response of excised hypocotyl explants from SLM 046 with different cytokinins (TDZ and BA) and different ages of seedling.

Materials and Methods

Plant materials and tissue culture

Brassica napus cv. SLM 046 was used as the experimental plant material. Seeds were sterilized and placed in germination medium (Takasaki et al 1997). Hypocotyls were excised from 7, 14 and 21-day-old seedlings. The hypocotyl sections were transferred to callus induction medium (CIM) contained 1 mgl^{-1} 2,4-D. After 7 days, they were transferred to the shoot regeneration medium (SIM) containing different concentrations of BA (1.5,3,4.5 mgl^{-1}) and TDZ (0,0.15,0 0.3 mgl^{-1}). Sections were subsequently transferred to fresh medium of the

same composition every two weeks. After 6 weeks, shoots were excised from calli and placed on a root induction medium (RIM) containing 2mgl^{-1} IBA. The rooted plantlets were grown in vermiculite for about 4 weeks before transferring into soil in the green house.

Experimental Design and statistical Analysis

Treatments consisted of three levels of BA concentration (1.5,3,4.5 mgl^{-1}), Three levels of TDZ concentration (0,0.15,0.3 mgl^{-1}) and three levels of seedling age (7,14,21 days) were arranged in a randomized complete block design, with three replications. Data were analyzed using MSTATC statistical software. Assessment of treatment differences was based on the 5% level of probability.

Results

The effect of plant growth regulator concentration on shoot regeneration (SR) was tested with a combination of BA and TDZ with 3 levels of explant ages on *B. napus* (table 1). The critical factor for SR was presence of BA in the medium. The maximum number of SR was obtained in presence of 3 mgl^{-1} BA. This agrees with a previous report by Ono et al. (1994). A Significant effect of TDZ was detectable at the 0.3 mgl^{-1} concentration. The age of explants also influenced SR. 21-day-old explants showed maximum number of SR. The less number of SR was 7 days explants. This observation was not consistent with the previous work (Takasaki et al.1997). Significant interactions of BA concentration and the age of explants were present the most number of SR obtained from 3 mgl^{-1} BA and 21 day old explants. The use of TDZ in combination with BA had no significant effect on SR. Although the most number of SR was 4.5 mgl^{-1} BA and 0.3 mgl^{-1} TDZ treatment. Interactions of the TDZ and the age of explants was no significant, but application of 0.3 mgl^{-1} TDZ with 3 levels of explant ages resulted in the most number of SR. Significant differences were found in interactions of the BA and TDZ and the ages of explants.

Table 1: Means of squares for regenerated shoots

Source of Variation	Degrees of Freedom	No. of Regenerated shoots
Replication	2	126.90 *
Age of Explant (A)	2	103.57 *
Benzyladenine (B)	2	1990.23 **
A X B	4	292.42 **
Thidiazuron (C)	2	990.48 **
A X C	4	43.36 ns
B X C	4	6.64 ns
A X B X C	8	43.05 ns
Error	52	26.60 ns

*, **: significant difference at 5%, 1%
ns: no significant difference

Discussion

The most number of regenerated plants was obtained by using of 3 mgl^{-1} BA, 0.3 mgl^{-1} TDZ and 21–day-old explants. Thidiazuron a substituted urea (N-phenyl-N'-1,2,3-Thidiazol-5-yl urea) with cytokinin activity has been as of more effective than BA for promotion of shoot proliferation (Mok et al., 1982). Christey et al. (1999) obtained the most SR from 0.1 mgl^{-1} TDZ and 10 mgl^{-1} BA. Cheng et al. (2001) by using of 0.55 mgl^{-1} and 4.5 mgl^{-1} BA obtained frequency of shoot regeneration about 90%.

Nielsen et al. (1995) has suggested that adenine - and phenylurea - type cytokinins have a common site of action in the plant cell. Both BA and TDZ can bind to a receptor, a cytokinin - binding protein (CBP). The CBP has two different binding sites. One site binds adenine-type cytokinins naturally, while the other is able to bind phenylurea - type cytokinins. Binding of an adenine-type cytokinin to CBP somehow induces the well-known cytokinin effects of shoot formation. When exogenous supply of TDZ, The binding of TDZ to the phenylurea CBP site may enhance the effect of BA or endogenous adenine - type cytokinin already bound to CBP.

TDZ, a substituted phenylurea is primarily used as a cotton defoliant and has been shown in various cytokinin bioassays to exhibit strong cytokinin-like activity similar to that of N6-

substituted adenine derivatives. The precise mechanism of action is as yet unknown. However, two hypotheses are presented. TDZ could directly promote growth due to its own biological cytokinins or it might induce synthesis or accumulation of endogenous cytokinins (Bretgane et al., 1994).

Also, cytokinins are inactivated irreversibly by oxidative cleavage of N6 side chain of the cytokinin substrate by cytokinin oxidase.TDZ is known as non-competitively inhibit cytokinin oxidase activity, thereby enhancing the availability of endogenous cytokinins (Eapen et al., 1998). Because cells within the same plant can have different endogenous levels of plant growth regulators and additional variation in receptor affinity or cellular sensitivity to plant growth regulator (Kim et al., 1997) it is reasonable to expect that in vitro responses will vary with this cultivar. Efficient regeneration of plants from rapeseed hypocotyl segments should facilitate genetic manipulation of this important crop. Several genes of agronomic value such as herbicide tolerance could be introduced into the rapeseed. Increasing of shoot regeneration rate in hypocotyl segments should be an important condition to the improvement of the processes of transformation *via Agrobacterium*.

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